Pestic. Sci. 1998, **52**, 75–80

Carbocyclic Coformycin: a Case Study of the Opportunities and Pitfalls in the Industrial Search for New Agrochemicals from Nature*

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(Received 4 February 1997; revised version received 13 May 1997; accepted 6 August 1997)

Abstract: The work undertaken to isolate the novel, herbicidally active compound, carbocyclic coformycin, to obtain sufficient quantities of the compound for full biological evaluation, and to identify its biochemical mode of action is summarised. Although the compound was extremely active against some weed species, limitations in its spectrum of activity precluded further development. Carbocyclic coformycin exerts its biological action through a novel mode of action by the inhibition of the enzyme adenosine 5′-phosphate deaminase (EC 3.5.4.6) following phosphorylation *in planta*. From this work, the potential benefits of natural product research in the discovery of new agrochemicals are highlighted along with some of the possible pitfalls. © 1998 SCI.

Pestic. Sci., **52**, 75–80, 1998

Key words: carbocyclic coformycin; herbicidally active natural product; adenosine 5′-phosphate deaminase (EC 3.5.4.6)

1 INTRODUCTION

Natural products can provide important inputs to the search for new agrochemicals in two main ways. First, natural products can provide new, biologically active compounds for either follow-up by classical chemical synthesis or direct exploitation through production by fermentation. Examples of both of the above are well documented in the literature, although it is fair to say that production of agrochemicals by fermentation is relatively rare. Second, even if the isolated compound cannot be directly exploited, further investigation can lead to the elucidation of the biochemical mode of action of the compound, which can then potentially be exploited further through biochemical design or biochemical screening.

The case history described here summarises the work undertaken with the novel, herbicidally active compound, carbocyclic coformycin, from its initial isolation to the efforts to exploit this discovery. From this, the potential benefits from natural product work are highlighted along with the possible pitfalls that can be encountered with this type of work.

* Based on a paper presented at the meeting 'Natural Products as a Source of Crop Protection Agents III' organised by L. G. Copping, B. P. S. Khambay and A. Mudd on behalf of the SCI Pesticides Group and the Royal Society of Chemistry and held at 14/15 Belgrave Square, London, on 9 & 10 December 1996.

2 INITIAL DISCOVERY

From our routine screening in the herbicide area, one particular broth showed very promising levels of activity. Of special note was the interesting symptomology. We had long ago learned to be suspicious of broths that showed only scorch activity as, in many cases, the activity had been lost when further testing was undertaken against larger and more robust plants than those used in the primary screen. However, this particular broth showed symptoms involving death of the meristematic tissue, implying systemic activity and was actually very reminiscent of the inhibitors of amino acid biosynthesis.

Based on this interesting symptomology, the broth was investigated further. However, chemical characterisation was hampered initially by the lack of a suitable bioassay to guide the purification and an in-vivo assay

 Pestic. Sci. 0031-613X/98/$17.50. Printed in Great Britain

involving the application of only a few microlitres of sample to the apex of *Polygonum lapthifolium* L. had to be developed. Despite the fact that this assay takes some three days to report, it proved to be very successful in guiding the initial purification. It quickly became evident that a number of nucleosides were associated with the active fraction, and extensive purification by HPLC was required to separate the individual components. The five compounds shown in Fig. 1 were eventually isolated and characterised, as described in detail previously.[1]

Two of these compounds, aristeromycin and adecypenol, have previously been reported in the literature.[2,3] The other three compounds, however, were novel but related to the known compound coformycin.[4] Compound **3** was characterised as the carbocyclic analogue of coformycin. Compound **4** was shown to be the stereoisomer of carbocyclic coformycin, termed here the 4′-epimer. Compound **5** was not fully characterised, but the evidence available suggested that it was a hexose conjugate to carbocyclic coformycin.

3 INITIAL BIOLOGICAL EVALUATION

Having isolated and characterised the above compounds, the next step was to determine their biological activity. Due to the small amounts of compound available at this time, only limited testing was possible, but this served to show that carbocyclic coformycin, **3,** was the most active compound (Table 1). Although the sugar conjugate of carbocyclic coformycin also showed good activity, we suspected that this was due to degradation back to free carbocyclic coformycin. Coformycin itself was also included in this test, but proved to have only very limited activity, despite being previously reported to be herbicidal.[5]

TABLE 1
Biological Activity of the Isolated Compounds

	Activity[b] *against*			
Compound[a]	*POLLA*[c]	*ABUTH*[c]	*ECHCG*[c]	*ALOMY*[c]
3	4	3	4	2
4	2	0	1	1
5	4	1	4	2
2	2	3	2	1
1	1	1	3	0
Coformycin	0	1	0	0

[a] Compounds were formulated in methanol + water (25 + 75 by volume) containing 'Tween' 20 (5 g litre^{-1}) and 'Pluronic' L61 (0·5 g litre^{-1}) and applied by hydraulic sprayer at 2000 litre ha^{-1} to give a final rate of 200 g AI ha^{-1}.
[b] Assessments were made two weeks after treatment on the scale: 0 (no effect), 1 (slight damage), 2 (moderate control), 3 (good control), 4 (complete control).
[c] POLLA: *Polygonum lapathifolium* L.; ABUTH: *Abutilon theophrasti* (L.) Medic.; ECHCG: *Echinochloa crus-galli* (L.) Beauv.; ALOMY: *Alopecurus mysuroides* Huds.

4 COMPOUND SUPPLY

Following the promising biological results obtained, further quantities of carbocyclic coformycin were

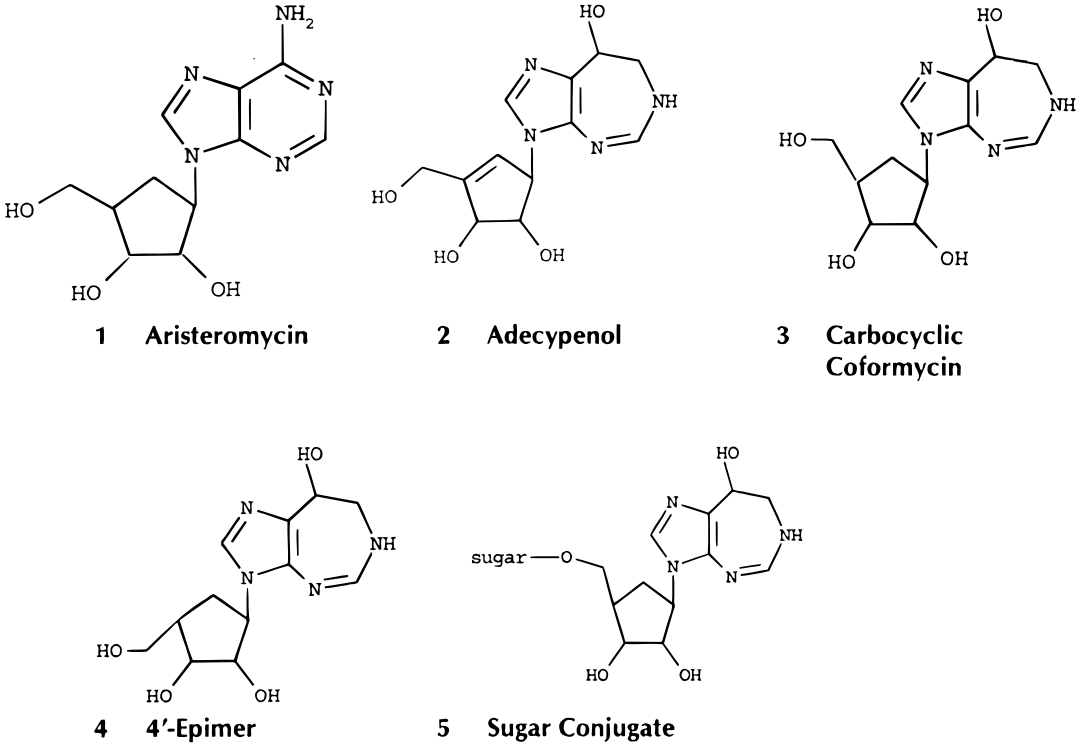

Fig. 1. Structures of the compounds isolated.

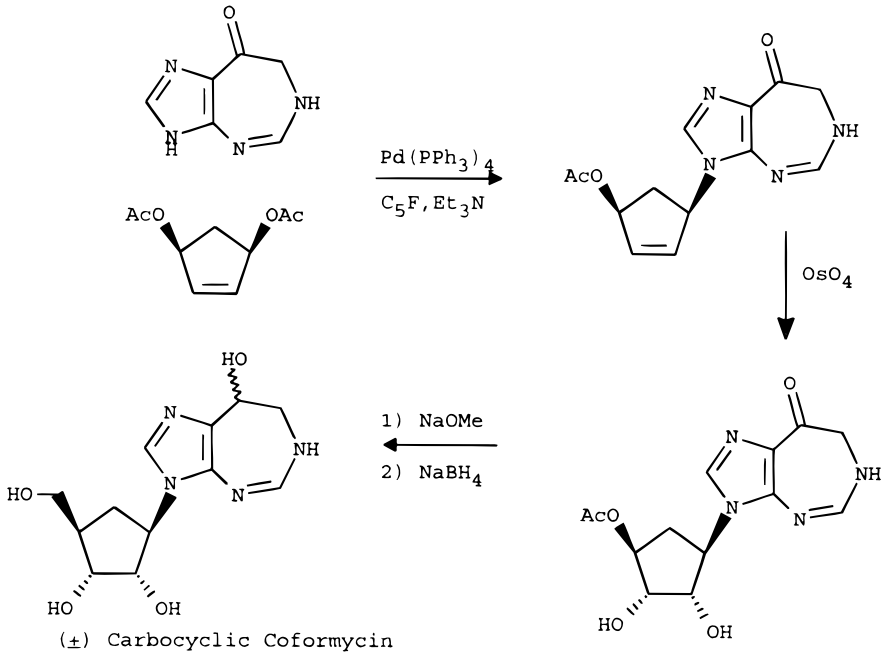

Fig. 2. Synthesis route to carbocyclic coformycin.

required to allow extended evaluation. Two approaches to obtain further compound, chemical synthesis and fermentation scale-up, were investigated.

4.1 Chemical synthesis

Synthesis of racemic carbocyclic coformycin was achieved using the route outlined in Fig. 2 and reported fully elsewhere.[6] While the synthesis did confirm the structural assignments for the natural product, only a few milligrams of compound could be obtained by this route. From this it was clear that synthesis would not be able to provide the required amounts of compound and we would therefore have to rely on the fermentation approach.

4.2 Fermentation scale-up

The titres of the compound in the initial fermentation were very low at around 1–2 mg litre^{-1} and the actual recovery was even lower. Initial work therefore concentrated on trying to improve the titres through the use of different media. For this work, a semi-quantitative HPLC assay was established using a Spherisorb SCX column eluted with 0·05 M $NH_4H_2PO_4$ at pH 2·6. Using this system, some 73 different media were investigated. Some improvement in titre to approximately 15 mg litre^{-1} was obtained with the best medium (Table 2). Unfortunately, no time was available to develop the fermentation conditions further and two

TABLE 2
Fermentation Medium Composition

Component	*Content (g litre^{-1})*
MOPS	21·0
EDTA	0·25
NaCl	0·5
$MgSO_4.7H_2O$	0·49
$CaCl_2.2H_2O$	0·029
Glycerol	23·0
Proline	11·5
K_2HPO_4	11·5
Trace salts	5 mg litre^{-1}

Adjusted to pH 6·5 pre-sterilisation.

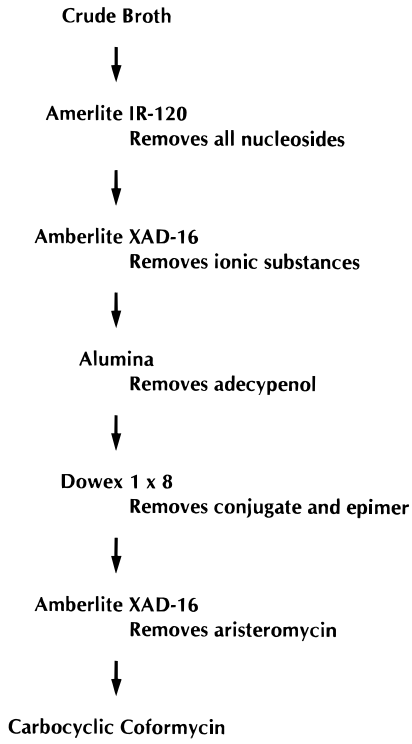

Fig. 3. Large-scale purification scheme.

large-scale fermentations, each of 5000 litres, were therefore undertaken. This yielded some 70 g of carbocyclic coformycin in approximately 12 000 litres of culture filtrate. After considerable development, the scheme outlined in Fig. 3 was subsequently used to process the crude extract. However, the large-scale purification proved to be more difficult than initially hoped, the key problems being in the initial volume reduction with Amberlite IR-120, where some 50% of the compound was lost, and the removal of the contaminating nucleosides which required extensive recycling. Despite these problems some 2·3 g of purified carbocyclic coformycin were obtained, at which point further work was halted to allow full biological evaluation.

5 EXTENDED BIOLOGICAL EVALUATION

Extensive glasshouse work was undertaken to evaluate the biological potential of carbocyclic coformycin. Standard glasshouse tests confirmed that the compound was extremely active, with a number of the species being controlled at rates as low as 2 g ha^{-1}. However, other species were relatively insensitive to the compound, even at rates as high as 500 g ha^{-1}, with the temperate grasses being particularly tolerant. Investigation of the effects on the major crop plants also showed unacceptable levels of damage at rates required to control the key weeds. From this work, we concluded that carbocyclic coformycin did not have the necessary spectrum of activity to be considered as a potential selective herbicide.

Further work was therefore undertaken to explore the potential of the compound as a non-selective herbicide by comparing its activity against glyphosate, and the results are summarised in Table 3. For more than half the species tested, carbocyclic coformycin matched the activity of glyphosate, despite being deliberately tested at only one-eighth of the rate. However, for the species against which glyphosate is known to have some weaknesses, carbocyclic coformycin was also only weakly active, and therefore did not appear to offer any real advantages over this commercial standard.

From the above data, we reluctantly had to conclude that carbocyclic coformycin did not have an appropriate spectrum of activity to warrant consideration for further development, and work on the natural product itself was therefore terminated.

6 BIOCHEMICAL MODE OF ACTION

Based on the potent activity of carbocyclic coformycin against a number of species, extensive work to elucidate the mode of action of the compound was undertaken. This work has led to the conclusion that the primary mode of action is the inhibition of the enzyme AMP deaminase (EC 3.5.4.6) following phosphorylation of the 5′ hydroxyl group on the carbocyclic ring *in planta*.[7] The key evidence leading to this conclusion is summarised below:

(i) Treatment of pea seedlings with carbocyclic coformycin led to a rapid and dramatic increase in the levels of ATP, indicating a perturbation in purine metabolism (Table 4).

TABLE 3
Comparison of the Biological Activities of Glyphosate and Carbocyclic Coformycin[a]

Species	*Inhibition of regrowth* (%)	
	Glyphosate (*1000 g* ha^{-1})	*Carbocyclic coformycin* (*125 g* ha^{-1})
Paspalum conjugatum Bergius	100	100
Cynodon dactylon (L.) Pers.	100	100
Sonchus arvensis L.	100	100
Sorghum halepense (L.) Pers.	99	75
Cirsium arvense (L.) Scop.	94	100
Elymus repens Gould	95	30
Cyperus rotundus L.	55	5
Convolvulus arvensis L.	35	35
Taraxacum officinale Weber	20	0

[a] Carbocyclic coformycin was formulated in methanol + water (25 + 75 by volume) containing 'Ethomeen' T25 (5 g $litre^{-1}$). Glyphosate was used as the commercial SL 'Round-up' containing 480 g glyphosate-isopropylammonium $litre^{-1}$. Both compounds were applied in water at 200 litre ha^{-1}. Four weeks after application, the foliage was removed close to the soil and the foliar regrowth was assessed after a further six weeks.

TABLE 4
Influence of Carbocyclic Coformycin on ATP, ADP and AMP Levels[a]

Time (*h*)	*% of untreated levels of*		
	ATP	*ADP*	*AMP*
1	121	109	104
2	196	104	94
4	226	87	100
6	284	121	97

[a] Seven-day-old *Polygonum lapathifolium* plants were sprayed to run-off with an aqueous solution of 100 μM carbocyclic coformycin and incubated in the light at 20°C. At the times indicated, the seedlings were harvested and immediately frozen in liquid nitrogen. Adenine nucleotides were extracted in 0·4 M $HClO_4$ at 4°C and analysed by HPLC.

(ii) This increase in ATP was preceded by a dramatic reduction in the extractable levels of AMP deaminase.
(iii) Carbocyclic coformycin was rapidly metabolised by pea seedlings to the 5′-phosphate analogue and this metabolite showed a strong, non-covalent association with the crude protein fraction that contains AMP deaminase.
(iv) Carbocyclic coformycin 5′-phosphate was found to be a potent, tight-binding inhibitor of AMP deaminase from pea seedlings with an I_{50} of approximately 20 nM in an in-vitro assay employing a 10-min pre-incubation period prior to the addition of the substrate AMP (640 μM).

These data provide very strong circumstantial evidence that AMP deaminase is the primary target for carbocyclic coformycin following phosphorylation *in vivo*. It is also interesting to note another precedent for the in-vivo phosphorylation of a herbicidally active natural product, hydantocidin, that also exerts its action through inhibition of purine metabolism, this time at the enzyme adenylosuccinate synthase.[8,9]

The inhibition of AMP deaminase following the application of carbocyclic coformycin has dramatic effects on adenylate metabolism, with a resultant uncontrolled increase in ATP levels and severe consequences for almost every aspect of the metabolism of the plant. Such effects can clearly explain why this enzyme should be such a potent herbicide target and this discovery opens the possibility of finding new herbicides through biochemical design or random biochemical screening.

7 CONCLUSIONS

The work described here clearly demonstrates the potential that natural products can provide in the search for new agrochemicals. The novel compound isolated was found to have extremely potent herbicidal activity and was not progressed further solely because its spectrum of activity did not match the commercial opportunities available at the time of the work. However, it did provide an interesting lead compound that prompted a relatively extensive synthesis programme to explore other analogues. In addition, the isolation of the compound has led to the identification of a novel biochemical mode of action that is being followed up.

However, natural product work is not without its problems. Perhaps the most significant are the problems in obtaining sufficient compound to allow the extensive testing that is required to determine the commercial potential of the isolated compound. In cases where large-scale fermentation is the only option to obtain sufficient compound, this will always require a much larger input than would be the norm for a standard synthesis project. Clear and well-defined milestones are essential for such work to be undertaken successfully and regular reviews are necessary to ensure that unjustifiable levels of resource are not consumed. However, unless the commitment is there to invest in this work, then the value of undertaking natural product research in the first place is somewhat questionable.

ACKNOWLEDGEMENTS

Many people were of course involved in this work and the author wishes to acknowledge the inputs made by all the colleagues not only at Chesterford Park but also at Glaxo Group Research and Schering AG in what was truly a multidisciplinary project.

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